Magnetic resonance spectroscopy investigations of brown adipose tissue and isolated brown adipocytes

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Abstract Brown adipose tissue and collagenase-isolated brown adipocytes were investigated in rats by means of ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. After chloroform–methanol extraction of brown adipose tissue, proton and natural abundance ^{13}C spectra of the chloroform fraction showed resonances attributable to triglycerides, and were qualitatively similar to those of the corresponding fraction of white adipose tissue. By means of quantitative analysis of ^{1}H spectra, fatty acid unsaturation and polyunsaturation in triglycerides were found to be lower in brown than white adipose tissue; moreover, unsaturation parameters decreased in triglyceride fatty acids of brown adipose tissue upon norepinephrine administration or cold acclimatization of rats, and were affected by the age of donors. The molar percentage of mono- and polyunsaturated C_{18} fatty acids in triglycerides was determined from ^{13}C spectra and found to change in the early post-natal period. Isolated, agarose-embedded brown adipocytes from 4-day-old rats showed a number of peaks in the carbohydrate region of ^{1}H spectra that were not present in spectra of white adipocytes and almost disappeared in brown fat cells of older animals. These peaks could be restored by insulin exposure. Natural abundance ^{13}C spectra of isolated brown adipocytes were resolved enough to allow unambiguous assignment of resonances to carbons of fatty acids, glycerol, glucose, ethanolamine, and choline. Calculation of the mono- to polyunsaturated fatty acids ratio in the cells was also performed. Nuclear magnetic resonance spectroscopy is a useful tool for the investigation of brown adipose tissue and adipocytes therefrom.—**Zancanaro, C., R. Nano, C. Marchioro, A. Sbarbati, A. Boicelli, and F. Osculati.** Magnetic resonance spectroscopy investigations of brown adipose tissue and isolated brown adipocytes. *J. Lipid Res.* 1994. **35:** 2191–2199.

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Two types of adipose tissue are found in mammals, including humans, the WAT and the BAT. The WAT has a major role in storage and release of fatty acid, and body thermal insulation. The BAT is a thermogenic tissue (1) that produces heat by oxidizing fatty acids in specialized, uncoupled mitochondria (2), thereby contributing to nonshivering thermogenesis (3). BAT activation depends on sympathetic (noradrenergic) stimulation. Nonshivering thermogenesis plays an important physiological role in the perinatal period, when other thermogenic mechanisms are still immature, as well as during the cold stimulus or at arousal from hibernation; in the adult, the BAT contributes to the diet-induced component of nonshivering thermogenesis. The interest in BAT (dys)function has steadily increased in the last 20 years because hypo- or hyperfunction of BAT has been involved in the pathogenesis of obesity (4, 5) and other pathological conditions (6, 7). The biochemistry and cell biology of BAT have been extensively investigated and excellent reviews are available (8, 9). However, characterization of BAT, specially in vivo, is far from complete.

In recent years, NMR has been a powerful tool in biological research. The NMR signal directly originates from the chemistry of the biological object of interest and the derived information can be used to produce both spectra (giving compositional data, NMR spectroscopy) and images (giving spatial localization of structures, MRI). Furthermore, spectroscopic data can be obtained in a spatially resolved manner in the living organism (topical NMR spectroscopy). In the first phase of a project aimed at NMR characterization of BAT, we used MRI at high spatial resolution to study BAT deposits in living laboratory rodents. Results demonstrated that MRI defines BAT deposits and their size in the intact animal, as well as morphological changes associated with norepinephrine

Abbreviations: BAT, brown adipose tissue; MRI, magnetic resonance imaging; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; TG, triglycerides; WAT, white adipose tissue; S/N, signal to noise ratio.

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administration and cold acclimatization (10, 11). Moreover, correlation was found between MRI signal and the histology and ultrastructure of BAT (12). The next exciting phase in NMR investigation of BAT would be studying the in vivo metabolism of the tissue in the intact animal by NMR. This should overcome difficulties found in biochemical studies, e.g., the relative unresponsiveness to hormones of isolated brown fat cells (13). As a first step toward in vivo NMR characterization of BAT, reference NMR data in tissue extracts and isolated brown adipocytes are worth obtaining. Tissue extracts are likely to give well-resolved spectra offering detailed information of BAT composition. Isolated cells will give information on the NMR visibility of cell constituents in a closer condition to the intact tissue. The effect on ^{1}H and ^{13}C NMR spectra of factors known to affect BAT metabolism and function was studied as well. For comparison, extracts of WAT or isolated white adipocytes were run in parallel when available.

MATERIALS AND METHODS

Animals

White (periepidydimal) and brown (interscapular) adipose tissue were taken under ether anesthesia from Sprague-Dawley rats fed standard rat chow and tap water ad libitum, and maintained under the conditions described below. The tissue was immediately used for preparation of tissue extracts or adipose cell isolation. Adipose tissues were taken from groups of 3-month-old rats maintained as follows: *a)* acclimatized at room temperature (about 20–23°C); *b)* acclimatized in the cold (2 weeks at 4°C); *c)* acutely injected twice in 6 h with norepinephrine (Arterenol bitartrate, Sigma, MO, 5 mmol in 1 ml saline intraperitoneally). Groups of newborn, 1-, 4-, and 10-day-old rats, all maintained at room temperature and given free access to maternal suckling, and 6-month-old animals were used in some experiments. NMR spectroscopy is very discriminative but relatively not sensitive, thus tissue from animals of the same group was pooled to obtain proper amount of material for analysis. To minimize the effect of variability among animals, only tissue from individuals of the same litter or reared under the same conditions (diet, temperature, etc.) was pooled.

Tissue extracts

Adipose tissue from 2 to 20 animals of the same group was pooled, cut in small (25–50 mg) fragments in Dulbecco's medium modified with Earle's salts (Gibco, Scotland, UK), and frozen in liquid nitrogen. Tissue fragments were then put in the extraction mixture consisting of chloroform–methanol 2:1 (v/v) and extracted essentially according to Folch et al. (14). The resulting clear chloroform phase was dried under a stream of anhydrous nitrogen gas and the dry matter was resuspended in deuterated chloroform and used for NMR analyses.

NMR was performed on a Varian VXR500S spectrometer operating at 500 MHz for ^{1}H and 125.69 MHz for ^{13}C. Proton NMR spectra were acquired with an acquisition time of 5 s (sweep width 5,000 Hz), a flip angle of 30°, and a total number of transients of 128. No line broadening was applied in data processing. The $CHCl_3$ proton signal at 7.27 ppm was used as chemical shift reference. Natural abundance ^{13}C spectra were acquired accumulating a total number of 8,000 transients with a repetition time of 22 s (acquisition time 20 s, delay 2.0 s); the sweep width was 25,000 Hz, the flip angle 45°. An inverse gated proton decoupling was applied to avoid NOE. Before Fourier transform a line broadening of 0.4 Hz was used in data processing. The $CDCl_3$ signal at 77 ppm was the internal chemical shift reference. All spectra were acquired at 25°C. The procedures for quantification of chemical parameters from ^{1}H and ^{13}C spectra are reported in the legends of Figs. 1 and 2. On the basis of duplicate samples, the coefficient of variation was 0.10–0.25% in ^{1}H and 1.7–1.9% in ^{13}C measurements, with the exception of linolenic acid, where coefficient of variation was about 8%, due to low concentration of linolenic acid in the samples and the relatively low S/N ratio.

Isolated cells

Adipocytes were isolated from WAT and BAT by the slightly modified method of Rodbell (15), apart from using Dulbecco's (Earle's salt modified) medium instead of Parker's. This method typically yields cells that are more than 95% viable by the Trypan blue exclusion test. After thorough washing in fresh, cold isolation medium, adipocytes were passed to a modified cold medium containing a double concentration of glucose (200 mg/dl, Fluka, Buchs, Switzerland) and bovine serum albumin (8%, Sigma), and rapidly mixed with an equal volume of 1% agarose (Sigma) dissolved in deuterium oxide (Merck, Darmstadt, Germany). The agarose-cell suspension was then quickly but gently extruded through a plastic cannula into 5-mm NMR tubes, allowed to gel, and immediately used for NMR spectra acquisition. In some experiments adipocytes from the same preparation were embedded in agarose as above in the presence or absence of insulin (100 μU/ml final concentration, Novo, Copenhagen, Denmark) and incubated for 1 h at 28°C before NMR spectra acquisition. Proton NMR spectra were acquired at 400 MHz on a Varian Unity 400; field-frequency lock was made on the deuterium signal of deuterium oxide. All the ^{1}H spectra were acquired with presaturation of water signal, using a presaturation delay of 1.5 s. For each sample sufficient free induction decays were accumulated to give a spectrum with a good S/N ratio. Data were taken using a sweep width of 6,400 Hz (acquisition time 3.75 s). The data were processed using standard

Varian software (without line broadening). The ^1H spectra are referred to the H_2O signal. Natural abundance ^{13}C spectra were taken at 75.43 MHz on a Varian VXR-5000-300 with a 42° pulse repeated every 1 s. For each sample sufficient free induction decays were accumulated to give a spectrum with a good S/N ratio. Data were taken using a sweep width of 18,000 Hz. The ^{13}C spectra are referred to an external standard (acetone). All spectra were acquired at 28°C.

Ultrastructural morphology

Aliquots of agarose-embedded adipocytes were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 45 min, postfixed for 1 h in 1% aqueous osmium tetroxide, and dehydrated in graded concentrations of acetone. Adipocytes were then embedded in an Epon/Araldite mixture and thin-sectioned on a Reichert Ultracut E ultramicrotome. Sections were placed on nickel grids, stained with lead citrate, and viewed in a Zeiss EM 10 electron microscope operated at 60 kV.

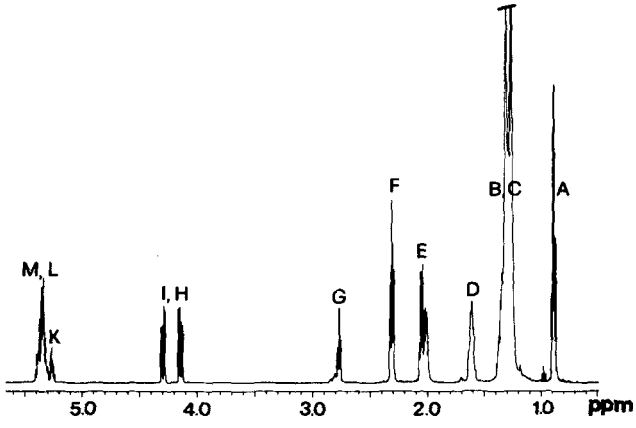


Fig. 1. Proton NMR spectrum of the chloroform (TG) fraction of BAT at 500 MHz. BAT was from 4-day-old rats. Spectra were acquired as indicated in Materials and Methods and were qualitatively similar in all experiments with both BAT and WAT. Resonances are assigned to protons of fatty acid chains and glycerol which are underlined and identified by letter (A-L) below:

A B,C E M,L G M,L E B,C D F I,H
$\underline{CH_3}\text{-}(\underline{CH_2})_n\text{-}\underline{CH_2}\text{-}\underline{CH}\text{=}CH\text{-}\underline{CH_2}\text{-}\underline{CH}\text{=}CH\text{-}\underline{CH_2}\text{-}(\underline{CH_2})_n\text{-}\underline{CH_2}\text{-}\underline{CH_2}\text{-}CO\text{-}\underline{CH_2}$
K
$R\text{-}CO_2\text{-}\underline{CH}$
$R\text{-}CO_2\text{-}CH_2$

In order to quantify chemical parameters in TG, the integer for each of the labeled resonances was automatically calculated by means of the standard Varian software implemented in the spectrometer and utilized in the following formulae. Calculation of mean chain length: $\Sigma/\frac{2}{3}A$; ($\Sigma = 2/3A + B + C + D + E + F + G + 2M + 2L$). Calculation of mean unsaturation: $(M+L)/\frac{2}{3}A$. Calculation of mean polyunsaturation: $G/\frac{2}{3}A$.

To exclude the presence of significant amounts of mono- and diglycerides the following two equations were verified: $A/(I+H) = \frac{9}{4}$; $F/(I+H) = \frac{6}{4}$.

TABLE 1. Unsaturation parameters of TG fatty acid acyl chains in BAT

Experimental Condition	Mean Unsaturation	Mean Polyunsaturation
Tr	0.97	0.38
CA	0.86	0.32
NE	0.86	0.29

Pooled BAT from young adult rats (n = 8-12) maintained at room temperature (Tr), acclimatized in the cold (CA), or acutely injected with norepinephrine (NE) was extracted in chloroform-methanol and the chloroform fraction was analyzed by ^1H NMR. The formulae used for calculation of fatty acids unsaturation parameters from ^1H spectra are reported in the legend of Fig. 1.

RESULTS

Tissue extracts

The ^1H spectra at 500 MHz of the chloroform fraction of BAT and WAT were qualitatively similar in all experiments. **Figure 1** shows a representative ^1H spectrum of the chloroform fraction of BAT. Assignment of resonances marked by letter is given in the figure legend. Resonances were exclusively attributable to fatty acid acyl chains and glycerol of TG in all the spectra of BAT and WAT. Mean TG acyl chain unsaturation and polyunsaturation in BAT was calculated from ^1H spectra according to the formulae reported in the legend of Fig. 1 and found to differ between BAT of rats acclimatized at room temperature and animals acclimatized in the cold or injected with norepinephrine (data summarized in **Table 1**); also, the age of the donor affected the degree of unsaturation of TG fatty acids (data summarized in **Table 2**). In pooled WAT extract of rats acclimatized at room temperature, mean unsaturation and polyunsaturation were 1.15 and 0.43, respectively. Mean fatty acid chain length (see legend of Fig. 1) showed a consistent tendency to be slightly lower in BAT than in WAT and to be slightly reduced in both tissues during cold acclimatization (data not shown). The number of fatty acid chains esterified with glycerol in adipose tissue TG was calculated as well (see legend of Fig. 1) and a mean value of 2.31 ± 0.18 (SD, n = 12) was found. **Figure 2** shows a representative natural abun-

TABLE 2. Unsaturation parameters of TG fatty acid acyl chains as a function of age

Age	Mean Unsaturation	Mean Polyunsaturation
Newborn	1.21	0.61
1 Day	1.13	0.50
10 Days	0.83	0.32
6 Months	1.09	0.39

Pooled BAT from rats (n = 12-20) of different ages was analyzed by ^1H NMR. See also Table 1.

dance ^{13}C spectra of the chloroform fraction of BAT at 125.69 MHz. Resonances (assigned in the figure legend) were attributable to carbons of the fatty acid acyl chains and glycerol of TG. Spectra of the corresponding fraction of WAT (not shown) were qualitatively very similar to those of BAT. The inset in Fig. 2 is an expansion of the region around 130 ppm where well-resolved resonances from double bond carbons of fatty acids are visible. The mol % of $C_{18:1}$ (oleic), $C_{18:2}$ (linoleic), and $C_{18:3}$ (linolenic) fatty acids were obtained from spectral data as shown in the legend of Fig. 2 and results are summarized in **Table 3.**

TABLE 3. Molar percentages of mono- and polyunsaturated C_{18} fatty acids of BAT TG in the perinatal period

Age	18:1	18:2	18:3
Newborn	43.8	53.2	3.0
1 Day	49.6	47.6	2.8
10 Days	38.5	59.0	2.5

Pooled BAT from 11-14 rats of different age was extracted with chloroform-methanol and the chloroform fraction was analyzed by natural abundance ^{13}C NMR. Calculations were made from ^{13}C spectra according to the formulae reported in the legend of Fig. 2.

Isolated cells

Proton NMR of 0.5% agarose in Dulbecco's modified medium showed the absence of significant resonance peaks in the range 0-7 ppm (spectra not shown). Spectra obtained from agarose-embedded adipocytes were generally stable for up to 5 h at 28°C (spectra not shown). **Figure 3** shows the ultrastructure of the cytoplasm in an agarose-embedded brown adipocyte. Cells were damaged to some extent during the isolation and embedding procedures; however, the general ultrastructure of the cell in Fig. 3 is good. In particular, note the well-preserved mitochondria with their typical, parallel cristae. Glycogen particles were scarce in isolated, agarose-embedded cells.

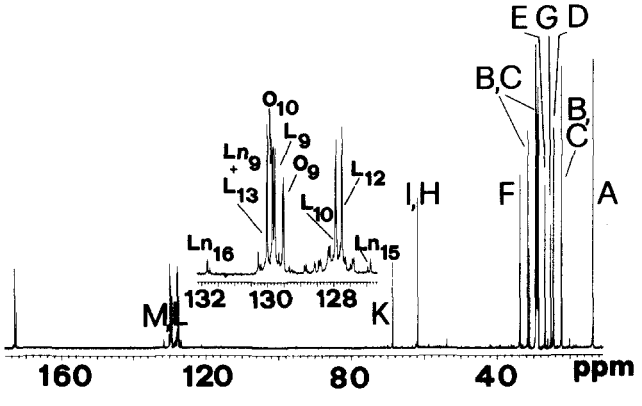
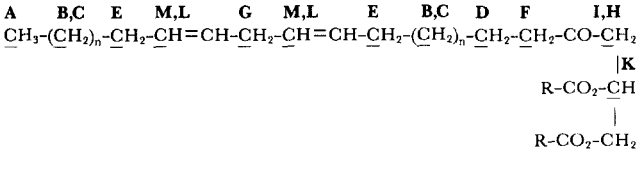


Fig. 2. Representative natural abundance ^{13}C NMR spectrum of the chloroform (TG) fraction of BAT at 125.69 MHz. The spectrum was acquired as indicated in Materials and Methods. Resonances are assigned to carbons of fatty acids and glycerol which are underlined and identified by letter below:



Inset: Expansion of the 127-130 ppm region showing well-resolved resonances from double-bonded carbons. Each peak is defined as O, oleyl (18:1); L, linoleyl (18:2); Ln, linolenyl (18:3) with the subscript corresponding to the carbon chain position. The mol % of fatty acids was calculated according to the following formulae (33): 18:1* = O_9; 18:2* = $(L_{12} + L_{10})/2$; 18:3* 0 $(Ln_{15} + Ln_{16})/2$; K = (18:1* + 18:2* + 18:3*); 18:1 = 18:1*/K; 18:2 = 18:2*/K; 18:3 = 18:3*/K.

Figure 4 (upper panel) shows representative ^1H NMR spectra at 400 MHz of collagenase-isolated, agarose-embedded brown (left) and white (right) adipocytes taken from 4-day-old and adult rats, respectively. Assignment of resonances is given in the figure legend. In brown adipocytes a number of well-resolved peaks were found in the 3-3.8 ppm region, which were not detectable in white fat cells and are assignable to carbohydrates. However, proton resonances from different carbohydrate moieties overlap in the 3-3.8 ppm region of ^1H spectra, and no assignment to specific carbohydrate molecules was made. In ^1H spectra from brown adipocytes of older rats, the peaks in the 3-3.8 ppm region were much less evident. Figure 4 (lower panel) shows ^1H NMR spectra of agarose-embedded brown adipocytes from 10-day-old rats in the absence of hormone (left) or in the presence of insulin (100 μU/ml) for 1 h (right). Apparently peaks in the region 3-3.8 ppm (insets) are barely detectable in the absence of the hormone and well-resolved upon insulin exposure. Insulin exposure did not associate with any significant changes of ^1H spectra in white adipocytes (spectra not shown). **Figure 5** shows representative natural abundance ^{13}C spectra at 75.43 MHz of agarose embedded brown (A) and white (B) adipocytes from 4-day-old and adult rats, respectively. Assignments of resonances for brown and white adipocytes are given in **Table 4** and **Table 5,** respectively, according to previously published results (16). Signals from glucose, ethanolamine, and choline carbons were only found in brown adipocytes. Signals from double bonded carbons were found near 130 ppm in the spectra of both white and brown fat cells. From the ratio of the intensities at 128.5 ppm (polyunsaturated fatty acids only) and 130.0 ppm (mono- and polyunsaturated fatty acids) the relative amounts of poly- to monounsaturated fatty acids in the cells can be obtained (16). The ratio was 0.6 in white and slightly lower in brown adipocytes.

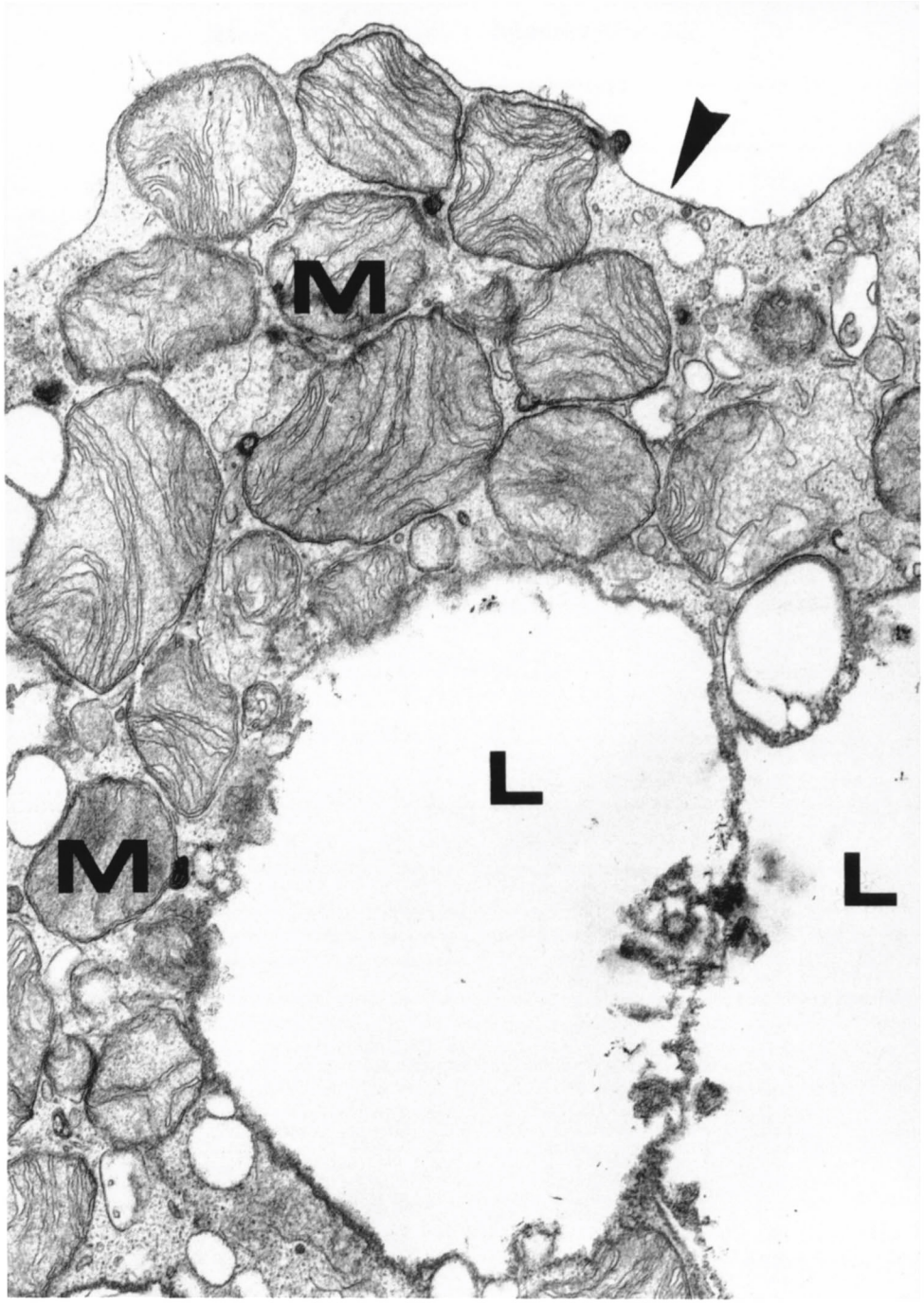


Fig. 3. Electron micrograph of an agarose-embedded brown adipocyte. The cell ultrastructure is well preserved. M, mitochondria; L, lipid droplet; arrowhead, cell membrane. Glutaraldehyde fixation. Original magnification ×10,000.

DISCUSSION

Previous investigations of BAT by means of NMR were scarce and rather limited in scope, showing higher spin-lattice relaxation time (T1) in BAT of cold-acclimatized rats versus controls acclimatized at room temperature (17) and indirect evidence of BAT thermogenesis by uncoupled proton conductance pathway (18). Results of the work presented here are the first extensive characterization of BAT and brown adipocytes by means of NMR. The following points have been demonstrated.

1) Age-, acclimation-, and norepinephrine administration-associated biochemical modifications of fatty acids are detected in BAT extracts by ^{1}H NMR.

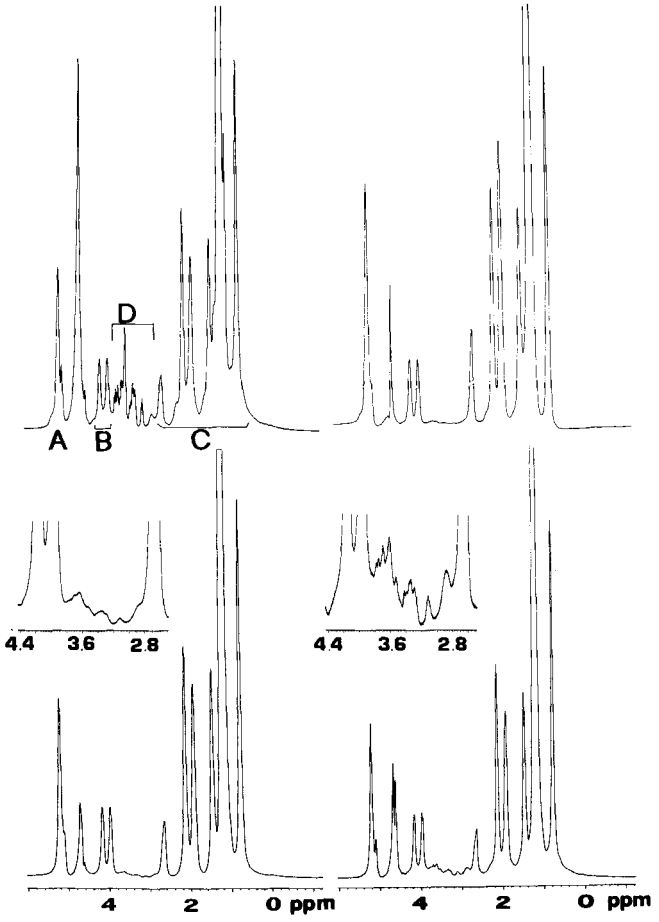
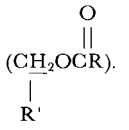


Fig. 4. Upper panel: Proton NMR spectra at 400 MHz of collagenase-isolated, agarose-embedded brown (left) and white (right) adipocytes from 4-day-old and 3-month-old rats, respectively. Comparison is made of neonatal BAT and adult WAT, because WAT is absent in the newborn rat. Spectra were acquired from about 5.7×10^5 white adipocytes and a similar number of brown fat cells. Resonances are assigned as follows: A, C protons of fatty acid chains (A, R-CH=CH-R'; C, CH_3-$(CH_2)_n$-R-CH_2-R'); B, protons of glycerol:



Note that in the 3-3.8 ppm region of the spectrum of brown adipocytes many peaks attributable to carbohydrate are found. The peak at 4.6-4.8 ppm is residual water. Lower panel: Proton NMR spectra from about 1.4×10^6 brown adipocytes in the absence (left) and in the presence (right) of 100 μU/ml insulin for 1 h. Compare peaks in the 3-3.8 ppm (carbohydrate) region (insets, ×4). These are well-resolved after insulin exposure.

2) Data on the relative amount of unsaturated fatty acids in the lipids are obtained from natural abundance ^{13}C spectra of BAT extracts and intact brown fat cells.

3) In ^1H spectra of isolated brown adipocytes, the effect of insulin exposure on the chemical composition of the cells is easily detectable.

4) Glucose, glycerol, ethanolamine, and choline are identified in natural abundance ^{13}C spectra of isolated brown adipocytes.

It should be clear that information derived from NMR investigations presented here is not original per se, as other analytical methods (e.g., gas chromatography) and cell metabolism studies were able to derive similar data previously. However, the work presented here was worth doing because we have now demonstrated, together with previous MRI results (10-12), that one methodology (NMR) is able to give detailed information on BAT over the whole span from chemical composition through isolated cells to in vivo function. Moreover, NMR investigations have many intrinsic advantages that cannot be disregarded. First, NMR identifies (and eventually quantitates), in the mmol range of concentrations, multiple molecules and metabolites in the same sample, thereby allowing simultaneous examination of a number of metabolic features in a single experiment with a minimum of sample preparation. Second, NMR procedures are not invasive and not destructive, therefore the same sample can be examined many times, serving as its own control.

Basic information on BAT lipid composition was offered by NMR analysis of the clear chloroform fraction of chloroform-methanol extracts of the tissue. Proton and ^{13}C spectra of this fraction showed resonances from exclusively neutral lipids in both BAT (Figs. 1 and 2) and WAT (not shown). These findings were expected, because TG is by far the major lipid fraction of adipose tissues and TG share the same chemical structure. Thanks to the good resolution achieved, despite the qualitative similarity

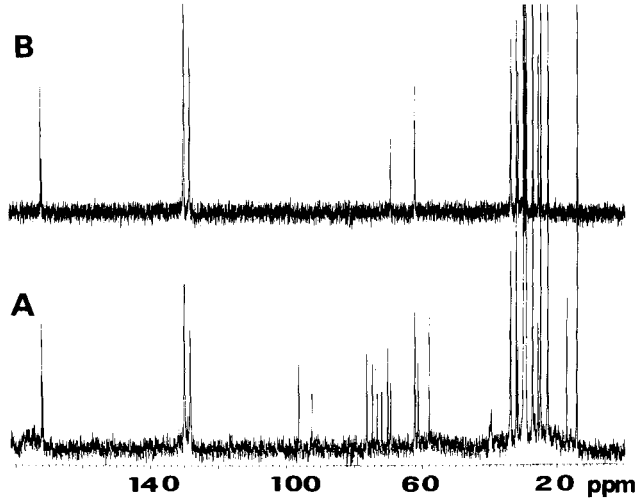


Fig. 5. Natural abundance ^{13}C spectra of collagenase-isolated, agarose-embedded white (B) and brown (A) adipocytes at 75.45 MHz. Spectra were acquired as indicated in Materials and Methods; a volume of 0.8 ml was typically assayed in a 5-mm NMR tube. The total number of scans was 4,800. Assignment of resonances (16) is given in Tables 4 and 5 for brown and white adipocytes, respectively. The peak at about 172 ppm is assigned to the carbonyl carbon of fatty acids.

TABLE 4. Assignment of natural abundance ^{13}C resonances from brown adipocytes

C		C	
171.86	-*COOR	60.95	C-6,βglucose
171.56	CH2CH2*CO	57.79	(*CH3)3N,choline
129.83	*CH=CHCH2CH =*CH-	40.4	*CH2NH2,ethanol
128.13	-CH=*CH- CH2*CH=CH	33.99	CH2*CH2CO, fatty acyl chain
96.25	C-1,βglucose	33.82	CH3CH2*CH2,
92.42	C-1,αglucose	32.19	fatty acyl chain
~79	C-2,βglucose	31.74	(*CH2)n,
76.23	C-3,βglucose	30.00	fatty acyl
76.14	C-5,βglucose	29.85	chain
74.54	C-1,αglucose	29.65	
73.14	C-1,αglucose	29.38	
71.85	C-5,αglucose	27.35	-CH=CH*CH2- CH2-
71.73	C-4,αglucose	25.74	CH=CH*CH2CH =CH2-
69.97	C-4,βglucose	25.01	*CH2CH2CO-
69.15	C-2,glycerol	22.89	CH3*CH2CH2-
62.01	C-1,C-3,glycerol	17.16	
61.11	C-6,αglucose	14.12	*CH3CH2CH2-

Brown adipocytes were isolated with collagenase, embedded in agarose, and analyzed in the NMR probe at 75.45 MHz.

among ^1H spectra of BAT and WAT of all groups of rats used in the present study, interesting information could be obtained by means of quantitative spectral analysis on the mean unsaturation and polyunsaturation of fatty acyl chains of tissue TG (Tables 1, 2, and Results). A first result was that, in adult rats acclimatized at room temperature, mean unsaturation and polyunsaturation values were higher in WAT than in BAT. This is in accordance with previous findings in the rat, mouse, and wild species, showing a higher proportion of saturated fatty acids in BAT in comparison with WAT (19–23). Moreover, NMR spectroscopy showed that a reduction in the degree of unsaturation and polyunsaturation of fatty acids in the lipid deposit of BAT takes place in association with acute (norepinephrine injection) or chronic (cold acclimatization) stimulation of the tissue (Table 1). The finding of a saturation effect of cold acclimatization on BAT TG is in accordance with previous gas chromatographic investigations in cold-acclimatized rats (24, 25). Results presented in Table 2, showing higher fatty acid unsaturation parameters of BAT at birth and in adult rats in comparison with 1- and 10-day-old animals, deserve some comment. In newborn rats and adult animals maintained at room temperature BAT thermogenesis is scarce due to immaturity of the thermogenic mechanisms and lack of the cold stimulus, respectively. On the other hand, 1-day-old pups are able to start thermogenesis in BAT and 10-day-old rats have fully active BAT thermogenesis (26), as well as cold-acclimated rats. It can be concluded from the data in Tables 1 and 2 that BAT activation is associated with decreased mono- and polyunsaturated fatty acids in tissue TG of both postnatal and adult rats. The possible misleading effects of mono- and diacylglycerol in the extracts were excluded by the absence in ^{13}C spectra (Fig. 2) of peaks attributable to free glycerol ($C_{1,3}$, +0.8; C_2, +3.1 ppm downfield from the respective peaks of esterified glycerol); moreover, the carboxyl carbon of fatty acids exclusively resonates in our spectra at about 172 ppm, thereby indicating complete esterification with glycerol (27). Accordingly, the calculated degree of esterification of glycerol in our extracts was close to the theoretical value of 2.35 (see Results).

Natural abundance ^{13}C spectra of the chloroform fraction of BAT (Fig. 2) and WAT at 125.69 MHz were qualitatively comparable to extracts of isolated white fat cells (27) and pure triolein (28). Peaks were assigned to carbons of fatty acids and glycerol. Peak resolution was very good despite the low natural abundance of ^{13}C (about 1%) because of the great abundance of TG in the tissue. Resonances in ^{13}C spectra of BAT are distributed over a much wider ppm range than ^1H spectra and are accordingly better resolved (compare Figs. 1 and 2). Thus, provided that complete proton decoupling is obtained to avoid NOE, reliable quantitative data on the mol % of unsaturated fatty acids (127–132 ppm) can be obtained from ^{13}C spectra. Data in Table 3 show that the ratio of mono- to polyunsaturated C_{18} fatty acids was about 0.78 at birth. In the fully thermogenic BAT at 10 days the ratio was 0.63 (−19%); taking into account results of Table 2, showing reduction of fatty acid unsaturation parameters in thermogenic BAT of neonate rats, it can be suggested

TABLE 5. Assignment of natural abundance ^{13}C resonances from white adipocytes

C		C	
171.79	-*COOR	29.89	fatty acyl
171.43	CH2CH2*CO	29.73	chain
129.71	*CH=CHCH2CH =*CH-	29.46	
128.01	-CH=*CH- CH2*CH=CH	29.32	
127.94	-CH=*CH- CH2*CH=CH	29.26	
69.02	C2 glycerol	27.23	-CH=CH*CH2- CH2-
61.88	C-1,C-3,glycerol	25.63	CH=CH*CH2CH =CH2-
33.86	CH2*CH2CO, fatty acyl chain	24.89	*CH2CH2CO-
33.70	CH3CH2*CH2,	22.77	CH3*CH2CH2-
32.07	fatty acyl chain	22.65	CH3*CH2CH2-
31.61	(*CH2)n,	14.01	*CH3CH2CH2-

White adipocytes were isolated with collagenase, embedded in agarose, and analyzed in the NMR probe at 75.45 MHz.

that $C_{18:1}$ fatty acid, i.e., oleic acid, is a preferential substrate for metabolic activities in neonatal thermogenic BAT. From the above results it can be concluded that combination of ^1H and ^{13}C NMR spectroscopy yields useful information on the composition changes of BAT TG. ^1H spectra are quickly obtained and resolved enough to allow reliable quantitative determinations of chemical parameters; ^{13}C spectra are more demanding (essentially in term of acquisition time), but offer interesting additional information on unsaturated fatty acids.

In recent years NMR has been used to investigate the composition and metabolism of a number of different cell types (29). NMR investigation of isolated fat-storing cells was limited to ^{13}C spectroscopy of free-floating white adipocytes (27) and cultured, perfused, agarose-embedded 3T3L1 cells accumulating TG (30). Brown adipocytes were not investigated. In the current experiments brown adipocytes were immobilized in agarose with good morphological preservation (Fig. 3) but, as long-term metabolic experiments were not envisaged, perfusion procedures were not applied. Results presented here (Figs. 4 and 5) show that informative, complementary ^1H and ^{13}C spectra can be obtained from isolated, agarose-embedded fat cells. Fast-to-obtain ^1H spectra showing a good S/N ratio differentiate white and brown adipocytes by the presence in the latter of obvious signals in the 3–3.8 ppm region, which are almost undetectable in white adipocytes (Fig. 4, upper panel); these signals can be safely assigned to carbohydrates. This result is in accordance with biochemical (1) and comparative gravimetrical, morphological, and MRI studies (12) showing that in the fully thermogenic BAT the water (i.e., cytosolic) fraction of the tissue is high (about 60% of total), whereas the lipid (TG) fraction is largely prevalent in the non-thermogenic BAT and in white adipose tissue. Definition of the ^1H parameters differentiating BAT and WAT is important because ^1H NMR is able to measure signals from carbohydrate (and glucose therein) with much higher speed and sensitivity than ^{13}C NMR. Our results show that, in principle, it should be possible to make topical metabolic investigations of the adipose tissues in vivo by means of ^1H NMR, possibly in correlation with MRI (10–12). The results reported here, that insulin exposure markedly increases carbohydrate signals in brown adipocytes (Fig. 4, lower panel, inset) together with recent data by Gruetter et al. (31) showing detection, assignment, and quantification of glucose signal in ^1H NMR difference spectra of the human brain, make this possibility more realistic.

Natural abundance ^{13}C spectra of isolated brown adipocytes from 4-day-old rats (Fig. 5A) confirmed results obtained in ^1H spectra, showing signals from TG, glycerol, and many peaks in the 60–100 ppm region, which were assigned essentially to glucose. In ^{13}C spectra, well-resolved resonances at 40.4 and 57.8 ppm were found that could not be detected in ^1H spectra. These were assigned to ethanolamine and choline, respectively. Both ethanolamine and choline are incorporated in phospholipids of cell membranes; the presence of abundant mitochondria in brown adipocytes is a reliable explanation for the presence of these two well-resolved signals in natural abundance ^{13}C spectra. The possible use of NMR for studying phospholipid metabolism in brown adipocytes is presently under investigation in our laboratory. The signals in spectra of agarose-embedded white adipocytes were from TG and glycerol only (Fig. 5B) in accordance with results obtained in excised canine white fat (28) and white adipose tissue in situ (16), and in isolated rat white fat cells (27). Brown adipocytes may contain glycogen. The finding of a peak at about 100.5 ppm (C_1 of glycogen) would have unambiguously indicated the presence of this glucose polymer in brown adipocytes; such a signal was not detected in our spectra. Electron microscopy examination showed that glycogen particles were almost absent in agarose-embedded brown adipocytes, probably because of the stress suffered by the cells during the isolation procedures. Finally, it should be noted that data on the relative proportions of mono- to polyunsaturated fatty acids in TG obtained from ^{13}C spectra of isolated adipocytes were in good agreement with results obtained after chloroform–methanol extraction (see Results). Taken together, results obtained in isolated cells support the possibility of performing some general investigation of glucose and lipid metabolism in brown adipocytes (and potentially the tissue in situ) by combined ^1H and natural abundance ^{13}C NMR, reserving the more demanding procedure of feeding cells (or animals) with ^{13}C-enriched compounds (32) to targeted problems.

In conclusion, results presented here have shown that a number of biochemical parameters giving useful information related to BAT and brown adipocyte composition and function can be derived from combined ^1H and ^{13}C NMR analyses. The current findings will serve as reference for future in vivo topical NMR spectroscopy studies of BAT.

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